Mat. Res. Soc. Symp. Proc. Vol. 723 © 2002 Materials Research Society M1.4

Characterization of MIPs Using Heterogeneous Binding Models

Ken D. Shimizu
Department of Chemistry and Biochemistry
University of South Carolina
Columbia, SC 29208, U.S.A.

ABSTRACT

New methods are presented for characterizing MIPs. These methods address the problems of quantitatively comparing the binding properties of different MIPs. Heterogeneous binding models were applied to MIPs based on an exponentially decaying distribution known as the Freundlich isotherm. The Freundlich isotherm was found to accurately model the binding isotherm of the majority of non-covalently imprinted MIPs. Using this model the experimental binding isotherm can be fit in log-log form to a linear equation from which the fitting parameters can be used to plot a quantitative affinity distribution which is a plot of the number of sites with respect to the binding constant of those sites. Comparison of MIPs using this methodology allowed for simpler and more accurate assessment of the binding properties than by previous methods such as the limiting slopes analysese of curved Scatchard plots

INTRODUCTION

Molecularly imprinted polymers (MIPs) are highly crosslinked polymers that are synthesized in the presence of a template molecule.[1,2] Upon removal of the molecular template, a binding cavity is formed with affinity and selectivity for the original template molecule. MIPs compare favorably to other synthetic materials that can be tailored with recognition properties including synthetic molecular receptors and designer antibodies. For example, MIPs have high thermal and chemical stability, are easily and inexpensively synthesized, and are easily tailored with binding properties for almost any molecule of interest.

Clearly, the binding properties of molecularly imprinted polymers (MIPs) are their most important characteristic. The comparison of the binding properties of MIPs, however, is complicated by the heterogeneity in MIPs. Unlike antibodies or enzymes that have a single type of binding site with high affinity and selectivity, MIPs contain a wide variation of binding sites that span the range in terms of binding affinity and selectivity. This binding site heterogeneity diminishes the utility of MIPs in chromatographic applications by leading to poor resolutions, highly concentration dependent selectivity, and severe peak asymmetry.[3] Heterogeneity also complicates the quantification and comparison of the binding properties of MIPs. For example, a common method to characterize MIPs is using a Scatchard plot (Figure 1). A homogenous system would yield a straight line in a Scatchard plot. MIPs, however, typically have a curved Scatchard plot due to the heterogeneity of the underlying binding sites. The heterogeneity in MIP1 can be modeled by the limiting slopes method by two straight lines to the curve, yielding two sets of binding parameters corresponding to the high- and low-affinity sites. Complications quickly arise when using this analysis to compare two polymers. For example, the high-affinity sites of a second polymer (MIP2) were measured and found to have a lower binding constant than MIP1($K_a = 5 \times 10^4$ M^{-1} versus 1.0×10^5 M^{-1}) but at the same time a greater number of binding sites (N =16.0 μmol/g versus 4.0 μmol/g). From this comparison, it is unclear which is the better polymer: MIP1 or MIP2, because it is unclear which binding parameter is more important: the number of binding sites (N) or the association constant (K_a).

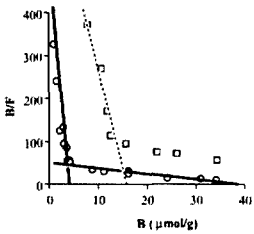

Figure 1. Scatchard plots of MIP1 (circles) and MIP2 (squares).

Table 1. Binding properties of high-affinity sites as measured by Scatchard analyses.

	MIP1	MIP2
N (μmol/g)	4.0	**16.0**
K (M^{-1})	**1.0×10^5**	5×10^4

Due to these difficulties, new methods were sought that might more clearly allow for the quantitative comparison of MIPs. First, it became clear that the number of binding sites and the association constant were not independent parameters but are dependent variables in heterogeneous systems such as MIPs. Thus, the question is not 'what is the association constant of an MIP?' Rather, the more appropriate question is 'what is the number of binding sites having a particular association constant?' This led to the use of affinity distributions that plots the number of binding sites (N) with respect to their association constants (K) to characterize MIPs. We have quantitatively measured the affinity distribution for a wide range of MIPs and found that MIPs can be well modeled by an unimodal exponentially decaying Langmuir-Freundlich distribution (Figure 2). In the case of the covalently imprinted polymer the peak of the distribution is in the analytical window and the exponentially; whereas, for the non-covalently imprinted polymer, only the exponentially decaying tail of the distribution is in the analytical window.

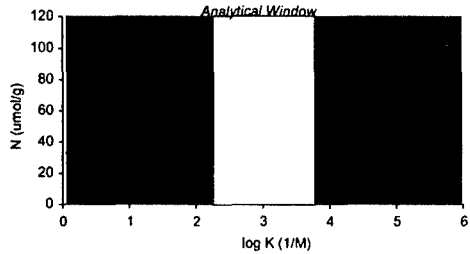

Figure 2. Affinity distributions calculated[4] for a covalently imprinted polymer[5] and non-covalently imprinted polymer.[6] The measured analytical window is shown as a gray box.

The relative positions of the distributions with respect to the analytical window appear to be fairly general as the examination of a wide range of non-covalent imprinted polymers from the literature were found to be accurately modeled by fairly simple exponentially decaying binding model, the Freundlich.[7] The appropriateness of the Freundlich isotherm in modeling the binding behavior of a MIP is easily graphically verified by plotting the binding isotherm in log bound (B) versus log free (F) format. Isotherms that conform to the Freundlich isotherm will fall on a straight line (Figure 3a). A linear regression fit of the isotherm to a log form of the Freundlich isotherm ($\log B = m \log F + \log a$), yields two fitting parameters a and m. These can be used to generate the corresponding affinity distribution using the new derived affinity distribution expression for the Freundlich isotherm ($N = 2.3\ am\ (1\text{-}m^2)\ K^{m}$) that relates the

number of binding sites (N) for each class of binding site having an association constant (K). The overall process is simpler than the Scatchard analyses, requiring only a single linear regression analysis, and generates an affinity distribution that more accurately characterizes the heterogeneous distribution of sites present in MIPs than the bimodal distribution of the limiting slopes Scatchard method.

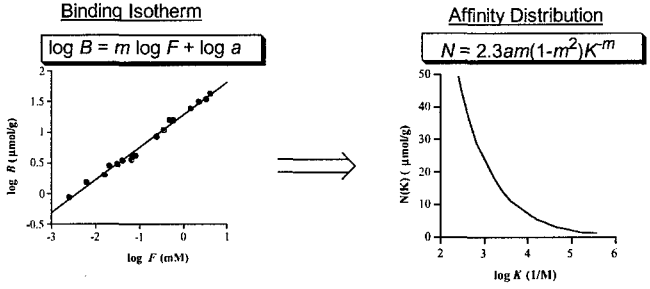

Figure 3. Binding isotherm (left) for an ethyladenine-9-acetate imprinted polymer in acetonitrile and the corresponding affinity distribution (right).

The ability to readily generate affinity distributions for MIPs, yielded the sought after method to quantitatively characterize MIPs that takes into account the heterogeneous distribution present in MIPs. In applying affinity distributions to characterize MIPs, there are a few practical limitations. First, the affinity distribution expression ($N = 2.3\ am\ (1\text{-}m^2)\ K^{-m}$) allows calculation of the distribution over the entire range of binding constants. However, in practice the accuracy of the calculated affinity distribution is limited by the concentration ranges of the binding isotherm. Typical boundaries are from $K_{min} = 1/F_{max}$ to $K_{max} = 1/F_{min}$.[8] Secondly, the application of an exponentially decaying distribution to model MIPs is only accurate with a given analytical window. The distribution cannot be globally accurate because 1) it would imply that there are an infinite number of binding sites (a physically impossiblility) and 2) at higher association (lower concentrations) it must reduce to Henry's law. Thus, in applying the Freundlich binding model to a given isotherm, it is necessary to first check if the Freundlich model actually accurately models the binding behavior over the measured concentration range. The easiest way to do this is graphically as shown in Figure 2. If the log-log plot of the binding isotherm does not fall in a straight line then the Freundlich isotherm cannot be used to model the binding behavior of the MIP over that concentration range. Either a different or more narrow concentration range can be selected or a different heterogeneous binding model must be used.[4]

The affinity distribution is commonly presented in two formats: 1) N versus log K and 2) log N versus log K. The first (N versus log K) is also known as the site energy distribution as log K is proportional to the binding energy (ΔG). In this format, the area under the distribution is the number of sites. This does not give the total number of binding sites rather the number of binding sites within a narrow range of association constants. In fact, the majority of MIPs have been measured in concentration ranges that do not allow for the accurate estimation of the total number of sites. The application of the Freundlich binding model only underscores this limitation. A second format for the affinity distribution (log N versus log K) allows for simple visual comparison of two polymers because the exponentially decaying distribution becomes straight lines. The slope (m) of the distribution, in this format, yields a measure of the ratio of

the number of high-affinity to low-affinity sites, with a flatter the slope corresponding to a higher percentage of high-affinity sites in the polymer.

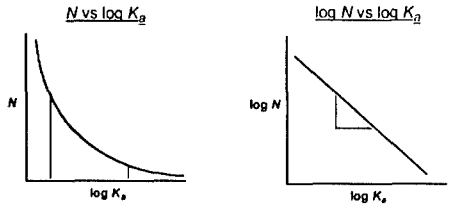

Figure 4. Representations of the two formats for the affinity distributions.

With the ability to readily characterize MIPs using affinity distributions, we were interested in the comparative distributions of imprinted and non-imprinted polymers. There are several different possibilities, shown in Figure 5. The imprinted polymer could have increased capacity and similar ratio of high- to low-affinity sites (Figure 5a), similar capacity but a more favorable ratio of high- to low-affinity sites (Figure 5b), or a combination of both (Figure 5c). A fourth possibility (not shown) is that either the imprinted or non-imprinted polymer do not conform to the exponential distribution.

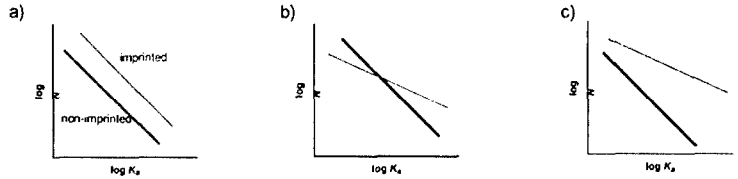

Figure 5. Representations of three different possible relative distributions for imprinted and non-imprinted polymers.

The MIP system selected for study was a well characterized non-covalently imprinted ethyl adenine-9-acetate (EA9A) selective system[4,6,7] which was based on a similar adenine selective MAA/EGDMA MIP reported by Shea *et al.*[9] The binding isotherms for the imprinted and non-imprinted polymers were measured from batch rebinding studies and all the polymers were found to be well modeled by the exponentially decaying distribution of the Freundlich isotherm. The comparison of the corresponding affinity distributions revealed that the imprinted polymers had higher capacity and ratio of high- to low-affinity sites in comparison to the non-imprinted polymer (Figure 6).

The three imprinted polymers differ in the concentration of template molecule (EA9A) in the polymerization mixture: (2.5 mM, 5.0 mM, and 12.5 mM). Both the capacity and percentage of high affinity sites improved as the concentration of template molecule was increased. In the case of the lowest concentration (2.5 mM EA9A) the molar monomer to template ratio was 120:1 and yet there was still a significant imprinting effect as seen by the much higher affinity distribution of the imprinted versus the non-imprinted polymer.

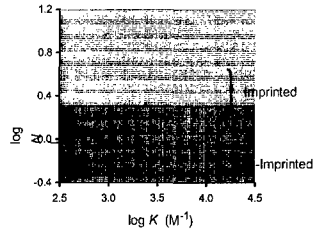

Figure 6. Measured affinity distributions for non-covalently imprinted and non-imprinted EA9A polymers. The imprinted polymers differ in the concentration of EA9A in the polymerization mixture: 2.5 mM (gray), 5.0 mM (broken), and 12.5 mM (solid lines), respectively.

Other variables in the imprinting process such as temperature (Figure 7a) and crosslinking percentage (Figure 7b) were also examined. Although these parameters have been extensively examined, the ability to quantitatively measure the breadth of the heterogeneity using affinity distribution analyses provided an opportunity to more accurately observe and understand the imprinting effect. Again, all the polymers were found to be accurately modeled by the Freundlich isotherm, yielding exponentially decaying distributions.

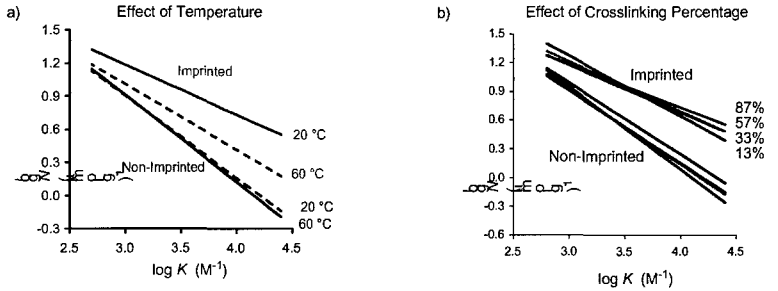

Figure 7. Overlaid affinity distributions of EA9A non-covalently imprinted and non-imprinted polymers that were synthesized under (a) differing temperatures and (b) crosslinking percentages.

Temperature had the more profound effect on the imprinting effect. The polymer imprinted under lower temperature conditions (20° C) displayed improved characteristics of higher capacity and ratio of high- to low-affinity sites in comparison the same formulation imprinted at higher temperatures (60° C). In contrast, the non-imprinted polymers synthesized at both temperatures were virtually identical in binding properties.

The effect of crosslinking percentage was examined by synthesizing polymers in which the crosslinking agent (EGDMA) has been substituted with increasing amounts of monomeric methyl methacrylate (MMA) reducing the overall crosslinking percentages without altering the concentration of functional monomer (MAA). The reduction of crosslinking percentage was expected to increase the flexibility of the matrix thereby decreasing the ability of the matrix to maintain the shape and affinity for the imprint molecules. Surprisingly, the crosslinking percentage has a relatively small effect on the affinity distributions. The polymer with 13%

through 87% crosslinking agent still showed a significant imprinting effect as seen by the higher affinity distributions of the imprinted versus imprinted polymers. Unlike changes in temperature and concentrations of template molecules, imprinted polymers with differing crosslinking agents all had similar numbers of binding sites. The polymers, however, were differentiated in the percentage of high-affinity sites. The affinity distribution of the 87% crosslinking agent MIP had the flattest slope and therefore the highest percentage of high-affinity sites. This study reveals some advantages of the affinity distribution analyses and helps explains some of the discrepancies in the literature on the effects of crosslinking percentage. If the polymers were compared at higher concentrations (low-affinity sites), then the 13% crosslinked polymer would be the best; whereas, if the polymers were compared at lower concentrations (high-affinity sites), then the 87% crosslinked polymer would be the best.

The heterogeneous distributions observed in non-covalent MIPs has a number of consequences on the binding properties and ultimate utility of MIPs. First, the severe heterogeneity of the exponentially decaying distributions explains the strong concentration dependence on the affinity and selectivity of MIPs. The MIPs show poor binding properties at high concentrations and excellent binding properties at low concentrations. This heterogeneous distribution is not well suited toward chromatographic applications that are operative over a wide concentration range from high to low concentrations. This distribution is compatible with applications at low-concentrations such as in sensing, where the small number of high-affinity sites would dominate the binding properties. In fact, the exponentially tailing distribution ensures that there will be a small but appreciable population of high-affinity sites. Finally, we have seen that improvements in the imprinting process increase the ratio of high- to low-affinity sites, but at the same time they also increase the heterogeneity of the polymers. The slope of the affinity distribution in the log N versus log K format also is the heterogeneity index, with a flatter slope being more heterogeneous. This helps explains results seen in the chromatographic applications, where polymers with higher affinity do yield greater separation factors but from a practical sense are worse off because of much lower resolution factors due to severe peak asymmetry and broadening.

ACKNOWLEDGEMENTS

The authors would like to thank the NIH (GM62593) for funding and support.

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